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Dobrava Virus as a New Hantavirus: Evidenced by Comparative Sequence Analysis

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Dobrava virus, recently isolated from a yellow-neck mouse *(Apodemus flavicollis)*, captured in a northern Slovenian village where severe cases of hemorrhagic fever with renal syndrome were recognized, was shown by serology and restriction enzyme digestion of PCR-amplified gene segments to be related to previously recognized hantaviruses. To investigate further the relationship of this new isolate to other hantaviruses, a portion of the medium (M) genome segment of Dobrava virus was amplified by PCR and the nucleotide sequence determined. Comparing the nucleotide sequence with the same gene region of other hantaviruses revealed an overall homology of 41.7%. A phylogenetic tree based on pairwise sequence similarity clearly showed that Dobrava virus is genetically distinct, and probably represents a new virus in the genus *Hantavirus* of the family Bunyaviridae.

KEY WORDS: Hantaan virus, hemorrhagic fever with renal syndrome, Slovenia, nucleotide sequence, RT-PCR

INTRODUCTION

The genus *Hantavirus* is comprised of at least 6 genetically and antigenically related but distinct viruses: Hantaan (HTN), Seoul (SEO), Puumala (PUU), Prospect Hill (PH), Thailand (THAI), and Thottapalayam (THP) [Lee et al., 1985; Schmaljohn et al., 1985; Xiao et al., 1992]. Some of these viruses are etiologic agents of the human disease, hemorrhagic fever with renal syndrome (HFRS). Recently, a new isolate, designated Dobrava virus, was obtained from an yellow-necked mouse, *Apodemus flavicollis*, captured in Slovenia [Avsic-Zupanc et al., 1992], where severe cases of HFRS were identified. Preliminary serological studies and polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis demonstrated its relationship with the existing hantaviruses, suggesting that it might represent a new member of the genus *Hantavirus*, family Bunyaviridae [Avsic-Zupanc et al., 1992].

We now report the nucleotide sequence comparison of a PCR-amplified fragment from the M genomic segment of Dobrava virus with previously published sequences of other hantaviruses. Dobrava virus has found to be genetically distinct from other hantaviruses, adding further evidence that Dobrava represents a new hantavirus.

MATERIALS AND METHODS

Virus and Cell Culture

Dobrava virus was isolated from the lungs of an *Apodemus flavicollis* [Avsic-Zupanc et al., 1992]. The original specimens were collected from Dobrava village in Slovenia, where severe cases of HFRS were identified. Vero E6 cells were infected with the virus and processed for total cellular RNA extraction by using a guanidine isothiocyanate/cesium chloride method, as described previously [Xiao et al., 1991].

Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) and Direct Sequencing of the Amplimer

Two primer sets used in the initial PCR amplification, HanG2-1:2 and HanG2-3:4, were derived from the glycoprotein G2 encoding region of the Hantaan virus M genome segment [Xiao et al., 1991]. The sizes of the expected amplimers were 411 and 334 bp, respectively. RT-PCR reactions were done as described previously [Xiao et al., 1991]. Briefly, total cellular RNA was copied to cDNA with avian myeloblastosis virus reverse transcriptase, then amplified for 35 cycles with the *Taq* polymerase, in an automatic DNA Thermal Cycler (Perkin-Elmer-Cetus, Norwalk, CT).

The amplified product was subjected to preparative electrophoresis by using 1.2% low melting point (LMP) agarose gel (IBI, New Haven, CT) prepared with Tris-acetate-EDTA (TAE) buffer. The target band was cut out and DNA recovered by using the GENECLEAN Kit (BIO 101, La Jolla, CA). Ten microliters of double-stranded DNA (1.5 μg) was denatured with 2 μl of 2 N

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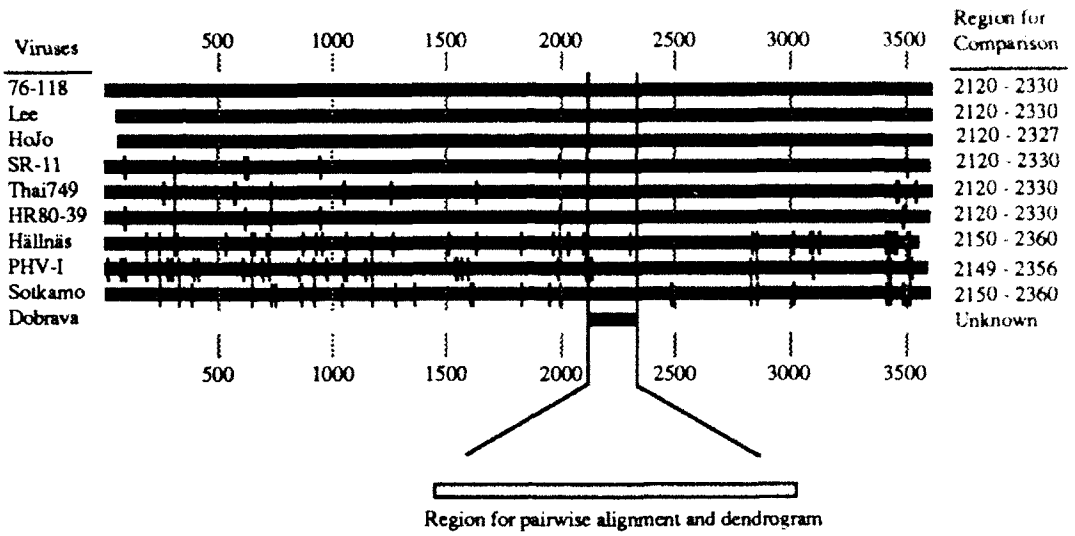

Fig. 1. Identification of cognate region for sequence comparison and dendrogram construction. Sequences were aligned against that of Hantaan 76-118 (Hash value = 6).

NaOH, ethanol precipitated, and dried briefly. Sequencing was done by the dideoxy chain termination method [Sanger et al., 1977], with the Sequenase Version 2.0 DNA Sequencing Kit from United States Biochemical (Cleveland, OH). Primers HanG2-1 and HanG2-2 were used for the sequencing reactions.

Sequence Analysis

Reference sequences were previously published and deposited in GenBank. Sequences examined included: Hantaan virus strains 76-118, Lee, and Hojo [Schmaljohn et al., 1987, 1988; Seoul virus strains HR8039 and SR-11 [Antic et al., 1991; Arikawa et al., 1990]; Puumala virus strains Sotkamo and Hällnäs-B1 [Giebel et al., 1989; Vapalahti et al., 1992]; Prospect Hill virus strain PHV-I [Parrington et al., 1991]; and Thailand virus strain Thai749 (Y-K. Chu et al., unpublished data). The Dobrava virus sequence was compared with all of these by the MacVector computer program from International Biotechnologies, Inc. (New Heaven, CT), to identify homologous regions in each sequence. The resulting sequence fragments were then compared with each other by using the CLUSTAL program of the PC/Gene software from IntelliGenetics, Inc. (Mountain View, CA). Pairwise similarity scores were calculated for all pairs of sequences and the degree of similarity used to form a tree (dendrogram) by clustering the sequences based on overall similarity.

RESULTS

Dobrava virus was successfully amplified with the HanG2-1:2, but not with the HanG2-3:4 primer pair, while both primer pairs amplified Hantaan virus strain 76-118 (data not shown). The amplimer generated with HanG2-1:2 was processed for sequencing. A sequence of 207 bp from the HanG2-1:2 amplimer was obtained and aligned against the whole M segment sequence of Hantaan virus strain 76-118, along with those of 8 other hantaviruses. The cognate region was identified for each sequence as shown in Figure 1, corresponding to the region 2120 to 2330 in the Hantaan virus cDNA sequence. This region was extracted from all the sequences for further pairwise sequence comparison by the CLUSTAL program.

Alignment of the 10 nucleotide sequences is shown in Figure 2. A dendrogram was constructed based on the calculated scores of pairwise similarity (Fig. 3). The length of compared region was 211 bp, with an overall identity of 88 bp (41.7%). To ensure the reproducibility of this dendrogram without Dobrava virus, another 500 bp region from the glycoprotein G1 encoding gene of the completely sequenced strains was analyzed with the same strategy. An identical dendrogram, excluding Dobrava virus, resulted.

DISCUSSION

Seroepidemiological investigations have identified at least three distinct antibody reaction patterns among HFRS patients seen in Yugoslavia and Slovenia [Avsic-Zupanc et al., 1990; Gligic et al., 1992]. These include reactions specific for a virus similar or identical to prototype Hantaan virus, a second pattern specific for Puumala virus, and a third pattern that appears to be Dobrava virus-specific. In addition, examining Dobrava virus with monoclonal antibodies to various hantaviruses demonstrated a unique serological reactivity pattern for this virus. Finally, PCR-RFLP comparisons of Dobrava and other recognized hantaviruses likewise found that this virus is related to, yet distinct from, all previously recognized hantaviruses [Avsic-Zupanc et al., 1992].

The results show that at the genomic level, Dobrava virus shared homology with other hantaviruses. However, it was clearly distinct when the sequence of the

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DOBRAVA     CCCATTAATCAAGAACAATCAGTAGATCTTCACATAGAGATAAGGAGTCA    50
HTN         CCACTTGAGGAAGCACAATCCATTGACCTACATATTGAAATAGAAGAACA    50
LEE         CCACTTGAAGAAGCACAATCCATTGATCTACATATTGAAATAGAAGAACA    50
HOJO        CCACTTGAAG---CACAATCCATTGATCTACATATTGAAATAGAAGAACA    47
SR11        CCAGTTAATGACCAACAGAGTGTCTCATTGCATATAGAAATTGAAAGTCA    50
SEOUL       CCAGTTAATGACCAACAGAGTGTCTCATTGCACATAGAAATTGAAAGTCA    50
THAI749     CCAGTTAACACAGAACAAGGAGTTCAATTACATATAGAGATTGAAGAGCA    50
HALLNAS     CCAGCAAACGAACAAGAGAAAATCCCATTTCATCTGCAGTTAAGCAAACA    50
SOTKAMO     CCGGCAAATGAACAAGAAAAAATACCATTTCATTTACAGATAAGTAAACA    50
PHV         ---GCAAATGAGCAAGAAAGAATCCCTTTTCACTTCCAGCTAGAGCGTCA    47

DOBRAVA     AGGATTTC-TACAAGTGTTCATGCATTAGGTCATTGGTTTGATGGA-GAC    98
HTN         GACAATTGGTGTTGATGTGCATGCTCTAGGACACTGGTTTGATGGTCGTC   100
LEE         AACAATTGGTGTAGATGTGCATGCTCTAGGACACTGGTTTGATGGTCGTC   100
HOJO        AACAATTGGTGTGGATGTACATGCTTTAGGACACTGGTTTGATGGTCGTC    97
SR11        AGGCATTGGTGCTGCTGTTCATCATCTTGGACATTGGTATGATGCAAGAT   100
SEOUL       AGGCATTGGTGCTGATGTCCATCATCTTGGACATTGGTATGATGCAAGAT   100
THAI749     AGGAATTGGTGCAGATGTCCATCACCTAGGCCATTGGTTTGATGCCCGGC   100
HALLNAS     AGTGATTCATGCAGAGATCCAGCATTTAGGTCATTGGATGGATGCTACAT   100
SOTKAMO     GGTGATTCATGCTGAAATTCAACATCTAGGCCACTGGATGGATGCTACAT   100
PHV         GGTGATTCATGCAGAGATTCAAAATTTAGGCCACTGGATGGATGGAACAT    97

DOBRAVA     TTAATTTAAAGACATCTTTTCATTGTTATGGTGCATGTACTAAGTATGAA   148
HTN         TTAACCTTAAAACATCCTTTCACTGTTATGGTGCTTGTACAAAGTATGAA   150
LEE         TTAACCTTAAAACATCCTTTCACTGTTATGGTGCTTGTACAAAGTATGAA   150
HOJO        TTAACCTTAAAACATCCTTTCACTGTTATGGTGCTTGTACAAAGTATGAA   147
SR11        TGAATCTAAAAACCTCATTTCATTGTTATGGTGCCTGCACAAAATATCAA   150
SEOUL       TGAATTTAAAAACCTCATTTCATTGTTATGGTGCCTGCACAAAATATCAA   150
THAI749     TTAATTTAAAAACATCATTCCATTGTTACGGGGCATGCTCAAAGTACCAG   150
HALLNAS     TTAATCTTAAAACTGCATTTCACTGCTATGGCTCATGTGAGAAGTATGCT   150
SOTKAMO     TTAACTTAAAAACTGCTTTTCACTGTTATGGTTCATGTGAAAAATATGCC   150
PHV         TTAATCTGAAAACATCATTCCATTGTTACGGTGCTTGTGAAAAGTATGCT   147

DOBRAVA     TATCCTTGGCATACAGCAAAATGCCACTTTGAAAGGGATTTCGAGTATGA   198
HTN         TACCCTTGGCATACTGCAAAGTGCCATTATGAAAGAGATTACCAATATGA   200
LEE         TACCCTTGGCATACTGCAAAGTGCCACTATGAAAGGGATTACCAATATGA   200
HOJO        TACCCTTGGCATACTGCAAAGTGCCACTATGAAAGGGATTACCAATATGA   197
SR11        TACCCATGGCACACTGCAAAATGCCATTTTGAGAAAGATTATGAGTATGA   200
SEOUL       TATCCATGGCACACTGCAAAATGCCATTTTGAGAAAGATTATGAGTATGA   200
THAI749     TACCCTTGGCATACAGCCAAGTGTCACTTTGAAAAAGATTATGAATATGA   200
HALLNAS     TATCCTTGGCAGACAGCAGGTTGTTTCATAGAAAAAGATTATGAATATGA   200
SOTKAMO     TACCCCTGGCAAACAGCAGGATGTTTCGTTGAAAAAGATTATGAATATGA   200
PHV         TATCCGTGGCAAACTGCAAAATGTTTCTTAGAGAAAGATTATGAATTTGA   197

DOBRAVA     GAACA--TGGG      207
HTN         GACGAGCTGGG      211
LEE         GACAAGCTGGG      211
HOJO        GACAAGCTGGG      208
SR11        AAATAGCTGGG      211
SEOUL       AAATAGCTGGG      211
THAI749     AAATAGTTGGG      211
HALLNAS     GACTGGTTGGG      211
SOTKAMO     AACTGGTTGGG      211
PHV         AACAGGGTGGG      208
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Fig. 2. Multiple sequence alignment of the homologous region. The dash signs represent gaps.

PCR amplified segment is compared with other hantaviruses. This finding, combined with previously published observations of serological uniqueness, suggests that Dobrava is yet another distinct member of the genus.

Construction of phylogenetic trees based on nucleotide sequence similarity has been used to depict relationships of closely related viruses as an alternative to the traditional serotyping of viruses. Rico-Hesse et al. [1987], comparing nucleotide sequences of 150 bp, showed that this method of comparison was able to establish epidemiologically consistent relationship among widely distributed isolates of wild poliovirus type 1 genotypes. From the dendrogram constructed in

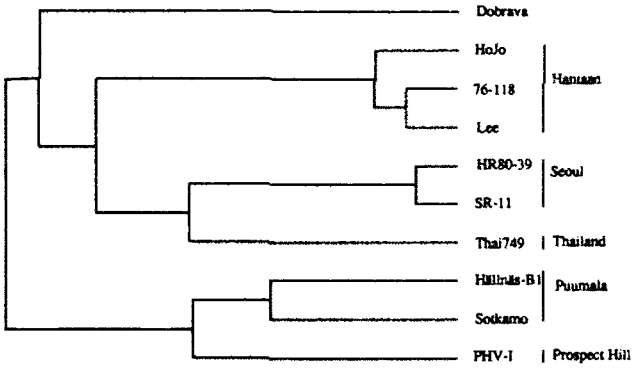

Fig. 3. Dendrogram based on the pairwise comparison of the hantavirus sequences. The horizontal distance to the node that connect 2 strains is proportional to the nucleotide sequence divergence between them.

this study, Dobrava virus was found to be most closely related to isolates of Hantaan, Seoul, and Thailand viruses; whereas it was more distantly related to isolates of Puumala and Prospect Hill viruses (Fig. 3). However, we showed it to be clearly separated from all of these viruses, in agreement with the serological results [Avsic-Zupanc et al., 1992]. Construction of a second dendrogram, based on the sequence analysis of a 500 bp segment from the G1 encoding region (excluding Dobrava virus), showed an identical relationship among these viruses, verifying the reliability of this partial sequencing analysis. In addition, similar relationships were recently reported by others who compared gene product sequences from the whole M segment of the hantaviruses with complete sequence information [Antic et al., 1992], further adding credence to our proposed placement of Dobrava virus.

The nucleotide sequence is the most reliable marker in identifying a virus; however, complete sequencing of all new isolates is neither practical nor necessary for this purpose. Data from this study showed that the overall interrelationship of hantaviruses documented from a region of approximately 211 bp is very similar (if not identical) to that from the whole M genome segment of hantavirus [Antic et al., 1992]. This provides a very useful and rapid tool for virus classification or typing, with the easy availability of purified DNA template generated by PCR.

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REFERENCES

Antic D, Lim B-U, Kang CY (1991): Molecular characterization of the M genomic segment of the Seoul 80-39 virus: Nucleotide and amino acid sequence comparisons with other hantaviruses reveal the evolutionary pathway. Virus Research 19:47–58.

Antic D, Kang CY, Spik K, Schmaljohn C, Vapalahti O, Vaheri A (1992): Comparison of the deduced gene products of the L, M and S genome segments of hantaviruses. Virus Research 24:35–46.

Arikawa J, Lapenotiere HF, Iacono-Connors L, Wang M, Schmaljohn CS (1990): Coding properties of the S and the M genome segments of Sapporo rat virus: Comparison to other causative agents of hemorrhagic fever with renal syndrome. Virology 176:114–125.

Avsic-Zupanc T, Likar M, Novakovic S, Cizman B, Kraigher A, van der Groen G, Stojanovic R, Obradovic M, Gligic A, LeDuc JW (1990): Evidence of the presence of two hantaviruses in Slovenia, Yugoslavia. Archives of Virology 115:87–94.

Avsic-Zupanc T, Xiao S-Y, Stojanovic R, Gligic A, van der Groen G, LeDuc JW (1992): Characterization of Dobrava virus: A hantavirus from Slovenia. Journal of Medical Virology 37.

Giebel LB, Stohwasser R, Zöller L, Bautz EKJ, Darai G (1989): Determination of coding capacity of the M genome segment of nephropathia epidemica virus Hällnäs B1 by molecular cloning and nucleotide sequence analysis. Virology 172:498–505.

Gligic A, Stojanovic R, Obradovic M, Hlaca D, Dimkovic N, Diglisic G, Lukac V, Ler Z, Bogdanovic R, Antonijevic B, Ropac D, Avsic-Zupanc T, LeDuc JW, Ksiazek T, Yanagihara R, Gajdusek DC (1992): Hemorrhagic fever with renal syndrome in Yugoslavia: Epidemiologic and epizootiologic features of a nation-wide outbrea in 1989. European Journal of Epidemiology (in press).

Lee PW, Gibbs CJ Jr., Gajdusek DC, Yanagihara R (1985): Serotypic classification of hantaviruses by indirect immunofluorescent antibody and plaque reduction neutralization tests. Journal of Clinical Microbiology 22:940–944.

Parrington MA, Lee P-W, Kang CY (1991): Molecular characterization of the Prospect Hill virus M RNA segment: A comparison with the M RNA segments of other hantaviruses. Journal of General Virology 72:1845–1854.

Rico-Hesse R, Pallansch MA, Nottay BK, Kew OM (1987): Geographic distribution of wild poliovirus type 1 genotypes. Virology 160:311–322.

Sanger F, Nicklen S, Coulson AR (1977): DNA sequencing with chain-terminating inhibitors. Proceedings of National Academy of Sciences of USA 74:5463–5467.

Schmaljohn CS, Hasty SE, Dalrymple JM, LeDuc JW, Lee HW, von Bonsdorff C-H, Brummer-Korvenkontio M, Vaheri A, Tasi TF, Regnery HL, Goldgaber D, Lee P-W (1985): Antigenic and genetic properties of viruses linked to hemorrhagic fever with renal syndrome. Science 227:1041–1044.

Schmaljohn CS, Schmaljohn AL, Dalrymple JM (1987): Hantaan virus M RNA: Coding strategy, nucleotide sequence, and gene order. Virology 157:31–39.

Schmaljohn CS, Arikawa J, Hasty SE, Rasmussen L, Lee HW, Lee PW, Dalrymple JM (1988): Conservation of antigenic properties and sequences encoding the envelope proteins of prototype Hantaan virus and two virus isolates from Korean haemorrhagic fever patients. Journal of General Virology 69:1949–1955.

Vapalahti O, Kallio-Kokko H, Salonen E-M, Brummer-Korvenkontio M, Vaheri A (1992): Cloning and sequencing of Puumala virus Sotkamo strain S and M segments: Evidence for strain variation in hantaviruses and expression of the nucleocapsid protein. Journal of General Virology 73:829–838.

Xiao S-Y, Chu Y-K, Knauert FK, Lofts RS, Dalrymple JM, LeDuc JW (1992): Comparison of hantavirus isolates using a genus-reactive primer pair polymerase chain reaction. Journal of General Virology 73:567–573.

Xiao S-Y, Yanagihara R, Godec MS, Eldadah ZA, Johnson BK, Gajdusek DC, Asher DM (1991): Detection of hantavirus RNA in tissues of experimentally infected mice using reverse transcriptase-directed polymerase chain reaction. Journal of Medical Virology 33:277–282.